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This grant entails two objectives. These objectives derived from prior work by ourselves and others which have identified TGF-β as an important cytokine in the biology of breast cancer. For established tumors, overexpression of TGF-β may result in increased in vivo tumor growth and metastatic spread. The first objective involves the analysis of resected breast cancer specimens, with the ultimate goal of determining the mechanism(s) by which TGF-β could be overexpressed by some tumors. There are as yet no definitive data or conclusions from this aspect of the work. The second objective of the grant was to identify the molecular determinants of promoter usage for TGF-β3 in breast cancer cells. These studies stem from our prior work in which we have characterized an alternative TGF-β3 promoter uniquely functional in breast cancer cells. We have made some progress in identifying differences in methylation pattern between cells in terms of their usage of the TGF-β3 promoter, and have identified the span of DNA sequence upstream of TGF-β3 which is responsible for the dual promoter usage.

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ANNUAL REPORT
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PI: Bradley A. Arrick, M.D., Ph.D.
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Introduction

This grant entails two objectives. These objectives derived from prior work by ourselves and others which have identified TGF-β as an important cytokine in the biology of breast cancer. For established tumors, overexpression of TGF-β may result in increased in vivo tumor growth and metastatic spread (Gorsch et al. 1992). The first objective involves the analysis of resected breast cancer specimens, with the main goal of determining whether overproduction of TGF-β in certain tumors is due to gene amplification (a common genetic alteration in tumor tissue) or some other mechanism, such as increased mRNA expression. The second objective of the grant was to identify the molecular determinants of promoter usage for TGF-β3 in breast cancer cells. These studies stem from our prior work in which we have characterized an alternative TGF-β3 promoter uniquely functional in breast cancer cells (Arrick et al. 1994).

Body of Report

First Objective (Objective A)

We had been focusing our efforts in working through the many difficulties which we are finding with regard to the reliable preparation from frozen tumor tissue intact mRNA as well as genomic DNA. The preparation of DNA has not been an unsurmountable problem. For this purpose, we have found that grinding the tissue, while still frozen (using liquid nitrogen as cooling source), and then doing an overnight 50°C digestion of the tissue fragments with a mixture of SDS, proteinase K, and EDTA (to inhibit DNase activity), followed by phenol-chlorofoam extraction and ethanol precipitation. This somewhat harsh treatment (which reduces the average size of DNA fragment so-obtained) is of no consequence for later PCR amplification. It is the preparation of high quality mRNA from samples which has continued to plague us. We cannot test, and therefore cannot rule out, the possibility that events which preceed our receipt of the specimen are responsible for the inconsistency we are experiencing. For instance, the amount of time the sample has spent "devitalized", and the temperature of preservation, are not strictly controlled for. At our medical center, mastectomy specimens are not routinely transported to the pathology department on ice, nor are they dissected and processed in the cold.

Progress towards the completion of this objective has fallen behind the timeline of the *Statement of Work* in the grant application We are currently proceeding with the analysis of the extracted genomic DNA from tumor specimens, testing primer pairs for the target gene TGF-β, as well as for a reliable "denominator" genetic locus. If we determine that gene amplification underlies the overexpression phenomenon, we will not need to test the RNA from these samples, and our difficulty in preparing the samples themselves will not hinder our ability to reach an ultimate conclusion for this aim.

Second Objective

The entirety of this objective is to understand the molecular basis by which breast cancer cells, unlike all other cell types examined by ourselves and others, utilize a different promoter for transcription of the TGF-β3 gene (Arrick et al., 1994). In this report, we will discuss our progress in the analysis of differences in methylation status at the CpG dinucleotides in proximity to the TGF-β3 promoters (Objective B-2), and our progress with transfections of chimeric TGF-β3 promoter plasmids (Objective B-3).

Experiments for Objectives B-1 and B-2

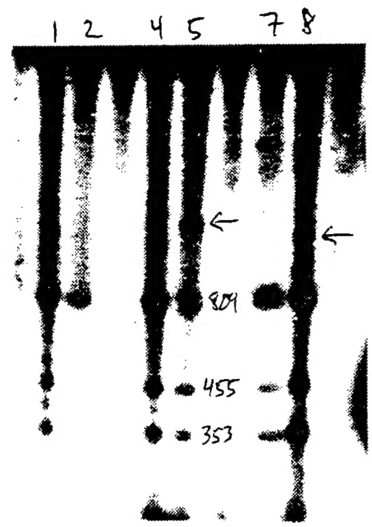
As detailed in our first report, we have not been successful with the experiments described under objective B-1 (DNase hypersensitivity assays). More specifically, when an interpretable result was obtained, there were no convincing differences between cells utilizing both TGF-β3 promoters and those which activate P1 only. This was more fully discussed in last year's report.

We have conducted experiments as outlined in the proposal looking specifically at the methylation status of HpaII/MspI restriction sites near the TGF-β3 promoters. Figure 1 contains one such experiment. In this experiment, genomic DNA from a breast cancer cell lines which utilizes both TGF-β3 promoters (SKBR3) and two which transcribe TGF-β3 mRNA from the upstream promoter (P1) only (HT-1080 and GBM405). As is outlined in Figure 8 of the original grant proposal, there are two HpaII/MspI sites between the two promoters, one downstream of P2, and a cluster of sites approximately 250-750 basepairs upstream of P1. The probe used in this Southern blot spans the downstream sites and extends to the 5' end of the cluster of sites upstream of P1. Three bands are evident from DNA digested with either enzyme from all three cell lines. These bands are approximately 353, 455, and 809 basepairs in length, and are the predicted bands for restriction enzyme digestion at the sites immediately surrounding the two promoters. In the HpaII lanes from the cell lines which do not transcribe from P2 there is a band evident at about 1.1 kb in length. This is best seen on the gel for HT1080 cells. There is no band of similar size in the HpaII-digested DNA from SKBR3 cells. This presumably reflects differences in methylation at those specific sites. This pattern can arise from either of two possibilities. One possible methylation pattern consistent with these results would be that the 5'-most sites within the cluster of sites are unmethylated in SKBR3 cells but methylated in HT1080 and GBM405 cells (in which case one or more of the remaining sites in this cluster are unmethylated in HT1080 cells and GBM405 cells). It is also possible that the upper band represents the combination of

the 809 and 353 bands, which would result if the MspI site closest to the downstream promoter transcription initiation site were partially methylated and therefore somewhat protected from digestion with HpaII (in cells not utilizing that promoter).

Figure 1.

Southern blot of genomic DNA following digestion with MspI or HpaII -- analysis of CpG methylation upstream of TGF-β3 promoters.



[Legend: DNA was isolated from SKBR3 cells (lanes 1 and 2), HT1080 cells (lanes 3 and 4), and GBM405 cells (lanes 5 and 6). Lanes 2, 4, and 6 were digestion with HpaII, the others with MspI. Arrows point to the bands unique to HpaII digested DNA from the cells which do not utilize P2. The 809, 455, and 353 bp bands are indicated.

Recent publications in the field of CpG methylation patterns have underscored the possibility (indeed likelihood) that at a given site there would be heterogeniety in methylation, even with a pure cell population. In other words, methylation could be strand specific, and typically when methylated a percentage other than 100% of DNA molecules are so-methylated. Our plan now is to proceed with genomic sequencing utilizing the bisulfite-modification method outlined in the original grant proposal. We will focus our attention on the areas of potential differential methylation suggested by the data such as in Figure 1.

Experiments for Objective B-3

The experiments for Objective B-3 relate to the transfection of cells with chimeric TGF-β3 promoter constructs. In our initial proposal, we provided data (Fig 6 on pg 46) which demonstrated that the DNA spanning the two transcription initiation sites (P1 and P2) had detectable promoter activity in the SKBR3 breast cancer cell line, but not in A673 or HT1080 cells, both of which do not use P2 for transcription of TGF-β3. We have prepared a series of related promoter plasmids which contain 5' or 3' deletions from this putative promoter. Figure 2 contains data from two independent transfection experiments utilizing this panel of plasmids with the SKBR3 cells. A relative activity of 1.0 was assigned to the plasmid containing no deletions (equivalent to the plasmid used in Fig 6 of the original proposal). Removal of the first 175 basepairs from the 5' end resulted in a little over a 5-fold decrease in promoter activity. Between 175 and 491 there is an evident negative element, since some increase in activity is evident with this more extensive deletion. Furthermore, it is apparent that the approximately 200 basepairs closest to P2 are not necessarily essential for transcription. See the brief discussion of objective B-4 for possible significance for the 5'-most 175 basepairs in the regulation of TGF-β3 expression.

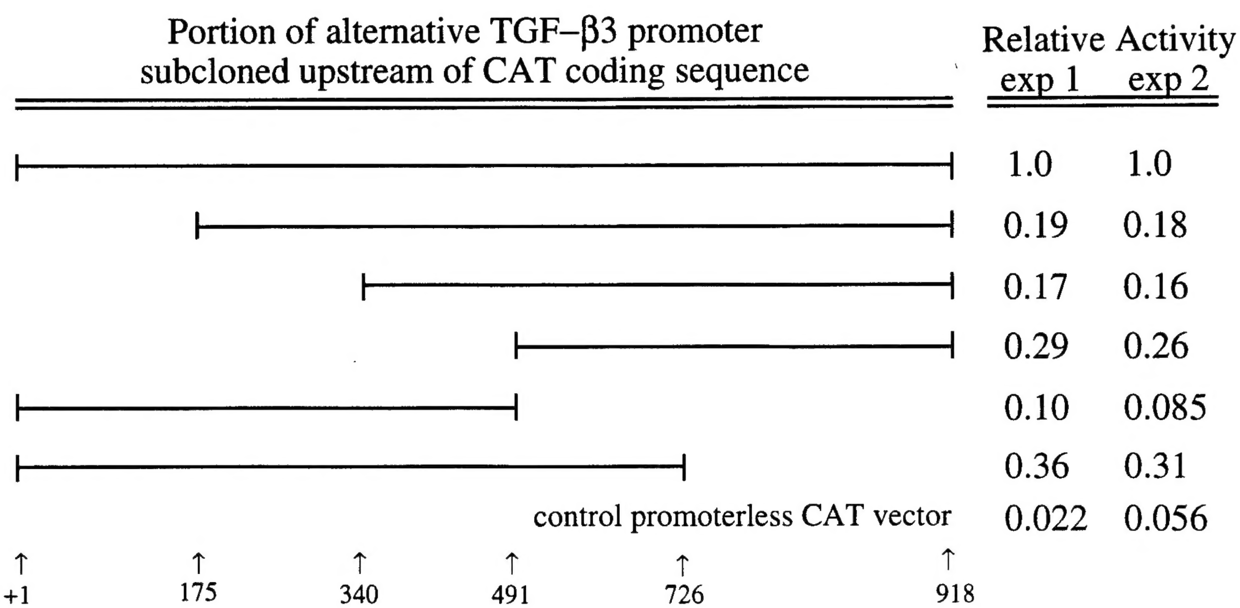


Figure 2.

In last year's report we briefly described that we had begun to prepare stable transfectants using plasmids in which the neomycin resistance gene is under the transcriptional control of the TGF-β3 promoter. G418-resistant colonies were grown up and mRNA isolated. This was then analyzed by Northern analysis, using a neo gene probe. In Figure 3A, from such clones of T47D and SKBR3 cells, we observed predominantly only one transcript species (actual size depends on the exact circumstances of integration into the host cell genome). We then tried a similar experiment with a plasmid in which the 5'-most 780 base pairs were deleted. This plasmid extends to the FspI site upstream of P1 (refer to diagram on Figure 9, pg 21 of the original proposal). Northern analysis of mRNA isolated from G418-resistant cells transfected with this plasmid (only SKBR3 was successfully transfected this time) demonstrated a recapitulation of the two transcript expression pattern of TGF-β3 in these cells (Figure 3B). This suggests that all of the "information" necessary for the utilization of both P1 and P2 in these cells is contained within this portion of DNA, and is not dependent upon other sequences upstream or downstream of the region included.

Because of the labor intense effort associated with the isolation and characterization of stably transfected cells, we will hold off on further experients of this sort until we define more clearly the methylation pattern differences between unipromoter cells vs bipromoter cells.

<u>Experiments for Objective B-4</u>

This objective relates to the identification of the estrogen-responive element(s) within the TGF-β3 promoter. Recent work from Yang et al. looking at the hormonal responsiveness of TGF-β3 in osteoblast cells has implicated a small segment of DNA (similar to the 5' most 175 basepairs identified in Fig 2) as important in the induction of TGF-β3 mRNA in these cells in response to estrogen or raloxifen (Yang et al, 1996). Once we have identified the minimal promoter sequence which regenerates the dual promoter expression pattern in breast cancer cells, we will use it, and the plasmid used in the experiment discussed above, to generate a stable transfectant clone from cells which express the estrogen receptor (e.g. T47D or MCF7). Unfortunelty, the SKBR3 cells we have generated do not express the estrogen receptor, and so cannot be used to ask questions related to the estrogen responsiveness of this promoter.

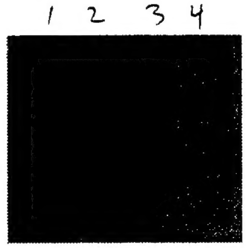
Figure 3A

Northern of stable clones

Legend: Lanes 1 and 2 are from T47D cells, lanes 3 and 4 are from SKBR3 cells. The upper "bands" actually represent cross-hybridization with the large ribosomal subunit RNA.

Figure 3B

Northern of stable clones



Legend: All four lanes are from separate SKBR3 clones. No signal was evident in lane 4, but the dual band pattern seen in lanes 1 and 2 was also evident in lane 3 with longer exposure.

Conclusions

With regard to the first objective of this proposal, no conclusions can yet be drawn regarding the incidence and mechanism of TGF-β1 increased expression in human breast cancer specimens.

Objective number two has revealed, in a qualitative sense, a difference in methylation in the region of the TGF-β3 promoter when breast cancer and non-breast cancer cell lines are compared. A more comprehensive mapping of sites of methylation in this region requires that we proceed with the technique of genomic sequencing of bisulfite-modified DNA.

Transient expression studies with chimeric promoter constructs in which the TGF-β3 promoter drives the CAT gene have suggested that there are both positive and negative elements within the first 918 bp of promoter sequence. Specifically, when numbered with nucleotide number 1 being at the site of transcription initiation from the originally-described TGF-β3 promoter, the region spanning 1-175 included a positive element, and a negative element was evident within the region spanning nucleotides 175-491.

Stable transfection studies with SKBR3 cells suggest that all of the elements that direct the utilization of the two TGF-β3 promoters could be contained within the region of DNA flanking these transcription initiation sites. In other words, distant enhancer elemants may not be required.

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